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(71) Applicant (for all designated States except US): THE
REGENTS OF THE UNIVERSITY OF CALIFORNIA
[US/US]; 9500 Gilman Drive, Mail Code 0910, La Jolla,
CA 92037-0910 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): WITZTUM, Joseph
[US/US]; 6912 Ofria Court, San Diego, CA 92093 (US).
TSIMIKAS, Sotirios [US/US]; 2802 Arnoldson Avenue,
San Diego, CA 92122 (US). PALINSKI, Wulf [DE/US];
4031-87 Porte Depalmes, San Diego, CA 92122-5150

(US). SHAW, Peter, X. [CA/US]; 10860 Caminito Arcada,
San Diego, CA 92122-5150 (US).

(74) Agents: MCCLAIN, James, W. et al.; Brown, Martin,
Haller & McClain LLP, 1660 Union Street, San Diego, CA
92101-2926 (US).

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(54) Title: REAGENTS AND METHODS FOR DIAGNOSING, IMAGING AND TREATING ATHEROSCLEROTIC DISEASE

(57) Abstract: The invention provides a novel human Mab Fab, cloned by phage display, and its use in diagnostic and therapeutic
methods. In particular the invention provides a method for analyzing the OxLDL components of atherosclerotic plaques in vivo and
a means to determine their relative pathology. As the method is based on a human Fab rather than a mouse Mab, the progress or
regression of the disease may be monitored over time. The antibody may also be used for the analysis of surgical or serum samples
ex vivo for the presence of OxLDL. The antibody may also be used to target therapeutic agents to the site of atherosclerotic plaques
or may have use as a therapeutic agent itself.

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REAGENTS AND METHODS FOR DIAGNOSING, IMAGING AND TREATING ATHEROSCLEROTIC DISEASE

CROSS-REFERENCES TO RELATED APPLICATIONS

5   This application claims the benefit of priority of United States provisional application Serial Number 60/161,493 filed 10/26/99 which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

10   *Field of the Invention:*

The invention herein relates to a novel human monoclonal antibody fragment (Fab), cloned by phage display that binds specifically to oxidized forms of low density lipoproteins (OxLDL) and not native LDL. More particularly, it relates to the use of the antibody for improved methods of diagnosis and

15   treatment of atherosclerosis.

Description of Prior Art:

Atherosclerosis is a chronic inflammatory disease that results from hyperlipidemia and a complex interplay of a variety of environmental, metabolic and genetic risk factors. The oxidation of low density lipoprotein (LDL) plays a

20   central, if not obligatory role, in the atherogenic process. Early studies showed that acetylation of LDL greatly enhanced its uptake by macrophges and that the uptake occurred via "scavenger receptors" which were distinct from the classical LDL receptor. Unlike most receptors, these scavenger receptors were not downregualted following uptake of OxLDL. Due to the excessive uptake of

25   OxLDL and its associated lipid by the macrophages, the cells obtained a characteristic foam-like appearance. The appearance of such cells is one of the first hallmarks of atherosclerotic disease. Foam cells accumulate within the intima (under the endothelial lining) of the vessel walls where they become unstable and plaques, the hallmarks of more advanced disease. Inflammatory

30   conditions develop leading to the development of complicated lesions.

There is much evidence that OxLDL contributes to atherogenesis by a number of mechanisms. The oxidation of polyunsaturated fatty acids in phospholipids of lipoproteins generates many breakdown products such as

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malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and other reactive moieties attached to oxidized phospholipids. Many of these intermediate products are highly reactive and can interact with lysine residues of associated proteins and phospholipids to generate various adducts. These adducts are known to occur
5   *in vivo* and are immunogenic. In murine models of atherosclerosis, such as apo-E deficient mice (ApoE$^{-/-}$) mice, atherosclerosis is correlated with the development of high titers of autoanitbodies to varous oxidation specfic epitopes of OxLDL. The consequences of such cellular and humoral responses are still poorly understood, but under certain conditions they can clearly modify the
10 natural history of the disease.

It is generally accepted that it is the composition of atherosclerotic lesions, in particular the content of lipids, OxLDL, foam cells, and smooth muscle cells, that determines their properties. Foam cells are often found in the sites of lesion that are susceptible to rupture. Activated macrophages recruited to clear
15 the apoptotic and necrotic foam cells, as well as OxLDL, secrete factors that weaken the plaque. Human pathology studies have shown that atheromas containing a large necrotic core, thin fibrous cap and large numbers of macrophage/foam cells in the shoulder are more predisposed to plaque rupture and thrombosis. These lesions, which frequently appear as mild or moderate
20 coronary stenoses in angiographic studies, are characterized pathologically as large atheroma with extensive lipid pools exceeding 40% of plaque areas. Angiography only provides a measure of arteial lumen, but fails to detect vessel wall pathology. Diagnostic methods that provide a measure of the overall extent of the atherosclerotic lesion, with an emphasis on OxLDL and lipid content,
25 would therefore be desirable. Moreover, the lipid core of atheromas can be assumed to contain extensive oxidized lipids that accumulated within foam cells and set free when cells undergo necrosis and apoptosis.

Non-invasive detection of atherosclerotic lesions is currently not clinically feasable. The gold standard for diagnosing atherosclerosis is angiography which
30 detects abnormal vessel lumen contours caused by encroaching atherosclerosis but does not directly identify abnormalities of the vessel wall. The widely recognized limitations of angiography include poor correlation with functional

stenosis, interobserver and intraobserver variability, underestimation of the
extent of disease because of diffusely atherosclerotic vessels, and arterial
remodeling. B mode and ultravascular ultrasonography can detect intima/media
thickening and calcification of vascular walls, but cannot clearly assess specific
5   tissue characteristics. Electron beam computed tomography detects only
calcium in vessel walls. Magnetic resonance imaging is still an investigational
tool for the detection of plaque components.

        Human studies have suggested that plaque rupture frequently occurs in
nonangiographically significant lesions that contain abundant lipid-laden
10   macrophages and large lipid pools within atheromas. Therefore imaging of
atherosclerosis directed at lipid-rich areas would be of value, not only in
detecting the extent of lesion burden, but also in the detecting clinically silent but
"active" lesions. Previous radioscintographic imaging agents have been limited
by poor specificity, low *in vivo* uptake in atherosclerotic plaque, and slow
15   elimination from the circulation, resulting in poor lesion/background ratios.
Various imaging agents have been used including radiolabeled LDL, fragments
of apolipoprotein B, autologous platelet and antiplatelet antibodies, non-specific
antibodies and Fc fragments, hematoporphyrin derivatives, and anti-malonic acid
monoclonal antibodies (Mabs).

20   OxLDL specific antibodies have been isolated from human and rabbit
atherosclerotic lesions which contain tightly bound IgGs that recognize epitopes
of OxLDL *in vitro* and stains atherosclerotic lesions *in vitro*. Mouse hybridoma
cell lines have been generated for the production of Mabs against OxLDL and
the antibodies were found to bind specifically to oxidized, rather than native
25   phospholipids. However all of the antibodies previously described were
monospecific, binding to only one form of OxLDL. The EO series of mouse
Mabs described by Palinski et al. (1996), were able to bind either OxLDL or
MDA-LDL, not both. Similarly, MDA2 and NA59, mouse Mabs described in other
studies, bind MDA-LDL and HNE-LDL respectively. Most importantly, these
30   mouse antibodies are limited in their usefulness for human applications *in vivo*
as they illicit an immune response that prohibits their repeated administration.

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Hybridoma technology, which is widely used in generating murine Mabs, is less successful in producing human hybridomas. Epstein Barr Virus (EBV) may be used to immortalize human lymphocytes, however due to the wide variety of neoepitopes in OxLDL, acquisition of human Mabs to many different
5   epitopes would be arduous. Furthermore, clones derived by this technique are frequently unstable and low secretors. Additionally, the EBV-transformants produce IgM antibodies, while anti-OxLDL antibodies can be both IgG and IgM isotypes.

Phage display combinatorial library technology provides a useful method
10 to generate human Mabs (Barbas and Lerner, 1991; Huse, et al., 1989). The libraries made from lymphocyte mRNA may consist of up to 10^8 recombinants of monoclonal Fab repertoires. By displaying the library on a filamentous phage surface and panning against a model epitope, monoclonal Fab antibodies can be selected and analyzed for their immunological properties and biological
15 activities. Fabs are ideal for use in both therapeutic and diagnostic methods as they can be produced in large quantities inexpensively and they are innately non-immunogenic. Additionally, they are not whole antibody molecules which can initiate a cascade of immune responses upon binding to their antigen.

20

SUMMARY OF THE INVENTION

The invention herein is the discovery of an antibody that binds to a novel epitope of OxLDL and MDA-LDL, but not native LDL, and its uses in imaging of atherosclerotic plaques, as a means for targeting therapeutics, and as a
25 therapeutic itself or model structure for the development of novel therapeutics for the treatment of atherosclerosis.

The invention is the discovery of a cloned human monoclonal Fab isolated from a phage display library generated from mRNA from peripheral blood mononuclear cells (PBMC) from a patient who was found to have high
30 antibody titers to MDA-LDL. After serial rounds of panning, a monoclonal IgG Fab antibody was isolated which bound specifically to both MDA-LDL and copper-induced OxLDL, but did not bind native LDL, as determined by both

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direct and competition binding assays. The Fab was found to bind specifically to atherosclerotic plaques, both *in vivo* and *in vitro* in human, mouse, and rabbit tissue. Furthermore, it was found to inhibit the uptake of OxLDL by macrophages, suggesting that the epitope on OxLDL defined by the Fab may be
5   an important ligand for the macrophage scavenger receptors in normal clearance or atherogenesis. We have named the Fab IK17.

Additionally, the invention overcomes the deficiencies of prior art detection methods for atherosclerotic lesions by the use of IK17. The invention describes a new method to non-invasively image the atherosclerotic lesions themselves by
10 the use of the use of a Fab conjugated to an appropriate molecule for detection. This further provides a means for particular discrimination of lipid rich components and oxidation rich components *in vivo*. The non-invasive nature of the imaging method using the invention reduces cost and risks to the patient allowing the method to be used as a means to monitor the effects of a treatment
15 regimen, as well as a primary detection method. The imaging method disclosed herein is more sensitive than previous methods allowing for the detection of atherosclerosis, both coronary and non-coronary, before the occurrence of significant stenosis, allowing for earlier intervention. It also provides a means for observing the vessel itself and assaying the amount of lipid present in the lesion,
20 providing a prognostic indicator and a method to grade the pathology of the lesion. It is a method to quantitatively monitor the effects of a treatment regimen as human antibodies will not induce an immune response. This type of surveillance cannot be performed with murine antibodies due to the potentially life threatening immune response to repeated administration of non-human
25 antibodies.

The invention allows for the improvement of current therapeutics and the development of novel ones for the treatment of atherosclerosis. The Fab provides a means for targeting therapeutic agents to the site of the plaques by covalently linking a thrombolytic agent, antioxidant, antimetalloproteinase or
30 other therapeutic agent to the antibody. Alternatively, IK17 itself, or small molecule analogs of IK17, could be used as drugs. IK17 is known to inhibit the uptake of OxLDL by macrophages, thus inhibiting the formation of foam cells.

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Inhibition of foam cell formation could decrease the deposition of lipids on the vessel wall and slow the progression of the disease.

DETAILED DESCRIPTION OF THE INVENTION

5   The invention herein is the discovery of a human monoclonal Fab that we have named IK17 that binds specifically to both OxLDL and MDA-LDL, but not native LDL, and uses of the Fab in the improved detection and treatment of atherosclerosis. This is the first discovery of an antibody that recognizes two forms of modified LDL. IK17 was isolated from a phage display library prepared

10   from RNA from PMNCs from a donor with coronary heart disease. It was found to be specific to Cu-OxLDL and MDA-LDL by a number of direct and competition binding assays using purified LDL. It was also found to be highly effective in a macrophage uptake assay, inhibiting the phagocytosis of both OxLDL and apoptotic cells. Additionally, the Fab was found to be useful for labeling

15   atherosclerotic plaques, both in vitro and in vivo. Radioactively labeled IK17 injected into mice was found to co-localize to atherosclerotic plaques as determined by Sudan staining.

         IK17 was cloned from a combinatorial Fab library by methods known to those skilled in the art. Briefly, human plasma samples were screened for the

20   presence of antibodies to OxLDL using a chemiluminescence assay (Hörkkö et al., 1996). A patient was identified as having a high antibody titer to MDA-LDL. PBMC were isolated from the patient and total RNA was extracted and used as a template to synthesize cDNA. The cDNA was used as a template for PCR amplification of the light and heavy chains, as described previously (Barbas and

25   Lerner, 1991). Subsequently, 3 pairs of extension primers were used for secondary amplification to add restriction sites to each of the three classes of fragments, V-kappa, V-lambda, and VH.

         PCR products of the expected size were cloned into the phage display vector pComb3H. The resultant phagemid DNA was transformed into XL-1 blue

30   E. coli cells by electroporation. Clones were panned against MDA-LDL coated onto an ELISA plates. A suspension containing approximately 10^9-10^{10} (100μl)

of recombinant phage was applied to each coated well and incubated at 37°C for 1 hour. After incubation, the wells were washed, once after the first round of panning or 10 times after subsequent rounds to remove unbound phage. Bound phage were eluted and used to infect bacteria for amplification by methods well

5 known to one skilled in the art. After the final round of panning, phagemid DNA was prepared to remove gene III which anchors Fab on the phage surface, by endonuclease digestion and religation. The resultant products were transformed into XL1-blue cells to express soluble Fab by induction with isopropyl beta-D-thiogalactopyranoside (IPTG). Cell lysates were prepared and ELISA assays

10 were performed to analyze Fab production and MDA-LDL binding activity. For subsequent experiments, selected monoclonal Fabs were purified using an IgG(Fab) affinity column by methods known to one skilled in the art.

Plasmid DNA containing the VH and VL genes of the Fab was isolated from cells and sequenced using an automated sequencer. Nucleotide

15 sequences were analyzed using the EMBL/GenBank database. Analysis revealed that the repertoire of Fab of the invention light chain uses a v-kappa 3 family gene (Vg/38κ/L6) with the rearrangement to Jκ2. The repertoire of heavy chain uses a VH3 family gene, 3-23/VH26c/DP47, with the rearrangement to JH4b.

20 The binding specificity of affinity purified Fab was studied by analyzing binding of the purified protein to MDA-LDL, Cu-OxLDL and native LDL as well as a panel of unrelated protein and nucleic acid antigens, using both direct binding and competition assays. The binding of the Fab was found to be specific to MDA-LDL and Cu-OxLDL, with a preference for MDA-LDL with an

25 affinity of 37nM. The Fab did not bind significantly to 4-HNE-LDL, nor did it bind to non-specific MDA modified proteins. The Fab was capable of binding both the lipid and protein fractions of the Cu-OxLDL, but did not bind the native LDL, either whole or fractionated

The ability of the Fab to localize to atherosclerotic plaques makes it ideal

30 for use in a method for detection of atherosclerotic lesions. The Fab can be produced easily and inexpensively in large quantities, as opposed to antibodies produced from hybridoma cell lines. Additionally, hybridoma lines may be

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unstable and decrease antibody expression levels over time. As there are no IgG type molecules in the *E. coli* in which the Fab is produced, purification can be carried out in a single affinity purification step. The antibody can be linked to radioisotopes, paramagnetic labels, echogenic liposomes, or other appropriate

5   agents that can be detected by imaging methods, and injected into the host intravenously. After an appropriate time, imaging can be performed, either whole body for diagnostic purposes or locally at specific sites, such as carotid artery, in a quantitative manner to assess the hosts response to a treatment regimen.

10   LDL and apoptotic cells accumulate at the site of atherosclerotic lesions and likely contribute to the pathology of the disease. However, they could be exploited as a means for targeting drugs to lesions. Drugs for the treatment of atherosclerosis could be targeted to the appropriate site by linking them to the IK17, which in turn binds to its unique, oxidation specific epitope in the lesion.

15   Such a method could be used to reduce the effective dose of drugs currently being used for atherosclerosis by targeting them to and retaining them at the site of lesions. Additionally it could be used to target therapeutic agents with desired activities that were found to be cleared to rapidly to be effective.

          As noted above, it was demonstrated that in animal models of

20   atherosclerosis, immunization with MDA-LDL could ameliorate the progression of the disease. IK17 could be administered as a protein or it could be administered using a gene therapy vector, as a means to ameliorate the progression of atherosclerosis.

          As the sequence of IK17 is cloned it could be easily manipulated for a

25   number of purposes. The coding sequence for linker amino acids, such as lysine or cystiene could be added for modification of IK17 with imaging or therapeutic agents. The pharmacodynamic properties of the antibody could be changes to increase stability, plasma clearance and tissue uptake. The sequences of the antigen recognition region could be mutagenized and

30   subjected to additional rounds of screening with phage display against different model compounds to identify other OxLDL binding antibodies.

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PREFERRED EMBODIMENTS

The preferred embodiments of the invention are described below. All publications mentioned herein are incorporated herein by reference to illustrate known methods and /or materials which may be of use in, but not essential to,

5   the practice of the invention.

Preparation of combinatorial Fab library and cloning of an OxLDL specific Fab

Plasma from patients was screened for the presence of antibodies to epitopes of OxLDL using a highly sensitive chemiluminescent immunoassay (Hörkkö et al., 1996). Antigens used for screening included Cu-OxLDL and

10   MDA-LDL as model epitopes and native LDL as a negative control. These antigens were prepared as described (Palinski et al, 1996). A patient who had serious coronary artery disease was identified as having a high antibody titer to MDA-LDL.

Peripheral blood mononuclear cells were isolated from the patient and

15   total RNA was isolated using RNA STAT-60 (Tel-Test) per the manufacturer's instructions. cDNA was synthesized with oligo dT primer using the Superscript II cDNA Synthesis Kit (Gibco-BRL). PCR reactions were carried out using the cDNA as template. Seventeen pairs of primers, including 4 pairs for immunoglobulin light chain of V-kappa genes and 5 pairs for V-lambda genes,

20   as well as 5 pairs for variable regions of heavy chain (VH genes), as described previously (Barbas and Lerner, 1991). Additionally, 3 pairs of extension primers were used for secondary amplification to add restriction sites to V-kappa, V-lambda, and VH.

PCR products of the expected size were cloned into the phage display

25   vector pComb3H by two steps: the V-kappa or V-lambda fragments were cloned into the SacI and XbaI sites of the vector first, followed by the cloning of the of the VH product into the XhoI and SpeI sites. The resultant phagemid DNA was transformed into XL-1 blue *E. coli* cells by electroporation. Clones were panned against MDA-LDL coated onto an ELISA plate. A suspension containing

30   approximately 10^9-10^{10} (100µl) of recombinant phage was applied to each coated well and incubated at 37°C for 1 hour. After incubation, the wells were washed, once after the first round of panning or 10 times after subsequent rounds, with

boric buffered saline (BBS) with 1% BSA. Bound phage were eluted with acetic acid (pH 2.0) and neutralized with 2M Tris buffer (pH 8.5). The eluent from each panning was used to infect bacteria for amplification by methods well known to one in the art.

5   After the final round of panning, phagemid DNA was prepared from infected bacteria. The DNA was digested with SpeI and NheI, gel purified, self-ligated, and transformed into XL1-blue cells.

Cultures were grown and Fab expression was induced with isopropyl beta-D-thiogalactopyranoside (IPTG). Cell lysates were prepared and ELISA
10   assays were performed to analyze Fab production and MDA-LDL binding activity. For subsequent experiments, selected monoclonal Fabs were purified against an IgG(Fab) affinity column by methods known to one skilled in the art.

Sequence analysis of anti MDA-LDL clones

Cultures were grown and plasmid DNA was isolated for sequencing using
15   an automated sequencer (ABI Prism). Nucleotide sequences were analyzed using the EMBL/GenBank database. Analysis revealed that the repertoire of Fab of the invention light chain uses a v-kappa 3 family gene (Vg/38κ/L6) with the rearrangement to Jκ2. The repertoire of heavy chain uses a VH3 family gene, 3-23/VH26c/DP47, with the rearrangement to JH4b.

20   *Macrophage OxLDL binding assay*

To confirm the capacity of the Fab to inhibit binding of OxLDL to macrophage scavenger receptors, a macrophage binding assay was performed. Mouse peritoneal macrophages were elicited by intraperitoneal injection fo 2ml of thioglycollate medium (Difco Laboratories) three days prior to harvesting the
25   cells by saline lavage. The macrophages were plate in 24-well clustered dishes at a density of 1 x 10^6 cells per well in RPMI 1640 supplemented with 5% fetal calf serum (FCS). Non-adherent cells were removed after three hours and the medium was replaced for overnight incubation.

The binding or degradation of ^{125}I-Cu-OxLDL to macrophages was
30   determined using methods of Goldstein et al. as previously described (Hörrkö, et al., 1999).

For the binding assay, cells were kept on ice for the entire procedure to prevent the internalization of the OxLDL. Cells were incubated at 4°C for 30 minutes in serum free media before the addition of ^{125}I-Cu-OxLDL in the absence or presence of competitors including non-radioactive Cu-OxLDL, a mouse
5 monoclonal directed against OxLDL, the Fab of the invention, and human IgG Fab. After three hours, cells were washed with ice cold PBS with 1% BSA and then solubilized in 0.2 N NaOH. Aliquots were removed for quantification of protein and radioactivity.

IK17 was able to inhibit the binding of the ^{125}I-Cu-OxLDL to the
10 macrophages efficiently (70-85%) in a dose dependent manner. The binding of ^{125}I-Cu-OxLDL was not effected by the presence of the non-specific human IgG Fab, demonstrating that the inhibition was specific and that IK17 could effectively mask the epitope on the OxLDL that was seen by the scavenger receptors.

Macrophage OxLDL degradation assay

15 Macrophages were harvested and plated by the same method as in the binding assay above. Specific concentrations of ^{125}I-Cu-OxLDL or ^{125}I-MDA-LDL in serum free media was added to each well in the absence or presence of competitors for 5 hours at 37°C. The amount of lipoprotein degraded was determined by the amount of ^{125}I-labeled trichloroacetic acid (TCA)-soluble
20 (noniodide) material present in the medium. IK17 inhibited the degradation of ^{125}I-Cu-OxLDL by macrophages by 50-65% in a concentration specific manner. The degradation of ^{125}I-Cu-OxLDL and ^{125}I-MDA-LDL were not effected by the presence of the non-specific human IgG Fab, demonstrating that the inhibition was specific and that IK17 could effectively mask the epitope on the OxLDL that
25 was seen by the scavenger receptors.

In both the binding and degradation assays, the amount of lipoprotein bound or degraded was calculated as per mg of cell protein and the result expressed as % of control in the absence of any competitor.

Binding of IK17 to apoptotic cells and inhibition of macrophage uptake.

30 FACScan: Dexamethasone treated apoptotic thymocytes were harvested and washed in ice cold PBS with 0.1% BSA. 1 x 10^8 cells were incubated with 50 µg/ml of IK17 or an isotype matched control human IgG(Fab) in PBS with

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0.1% BSA at 4°C for 20 minutes, washed and then incubated in for another 20 minutes with 10-20 µg/ml of propidium iodide (PI) and immediately analyzed by fluorescence activated cell sorting (FACS) analysis. PI staining allows for the separation of apoptotic and viable cells. The sorted cells were further analyzed

5 for their ability to bind IK17. IK17 was found to bind apoptotic cells, but not viable cells indicating that cells undergoing apoptosis display epitopes recognized by IK17.

Phagocytosis assay: Phagocytosis of apoptotic thymocytes was determined as described by Chang et al., 1999. Macrophages were elicited and

10 plated as described above. Cells were treated with dexamethasone and and suspended in 0.5 ml of PBS with 0.1% BSA and labeled with Calcein AM[R] from Molecular Probes for 15 minutes at 37°C. Cells were washed and resuspended in supplemented DMEM. To assess phagocytosis, labeled apoptotic thymocytes were added to macrophage containing wells in the absence or presence of IK17

15 or non-human Fab as competitor, and incubated at 37°C for 90 min. Wells were washed. Macrophages were harvested and fixed. Fluorescence was analyzed by FACS. Cells were sorted by size to select for macrophages and not smaller cells. Fluorescent labeling of macrophages indicated the uptake of the labeled apoptotic cells. These studies revealed that the uptake of apoptotic cells was

20 inhibited by 43% by IK17, indicating that IK17 is able to mask the epitope on apoptotic cells that is recognized by macrophage scavenger receptors.

Immunohistochemistry

Immunohistochemistry was performed on lesions of various stages from human and rabbit arteries. Sections from most of these tissues have been used

25 previously in a number of studies and characterized in terms of the presence of macrophages and oxidation specific epitopes. Tissues were obtained during surgery or autopsy and fixed, sectioned, and stained by methods known to one skilled in the art. Staining of atherosclerotic lesions in human and rabbit arteries indicated that the epitopes recognized by IK17 occur mostly in the necrotic core.

30 Macrophage-rich early lesions and shoulder areas of transitional lesions showed very little IK17 staining. Only a few human coronary lesions contained pockets

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of weak cellular staining. In contrast, strong IK17 staining was found in necrotic areas of advanced lesions of human coronary arteries and in the core of classical atheromas in human brain arteries. Similarly, in aortas from a rabbit model system of atherosclerosis, IK17 stained necrotic areas whereas only weak
5   staining of early lesions and superficial macrophages was detected.

These results are in contrast to staining patterns obtained with antisera and Mabs against oxidation specific epitopes that had been induced by immunization with homologous oxidized LDL or with natural Mabs cloned from atherosclerotic apo E⁺ mice. All of these antibodies consistently showed
10   stronger macrophage-associated and diffuse extracellular staining in early lesions in humans, rabbits, and mice with relatively weaker staining in necrotic areas.

Optimization of IK17 for use

The cDNA that codes for IK17 can be readily manipulated in a
15   randomized or directed manner to optimize it for use in imaging and other applications. Coding sequences for linkers to attach labeling reagents, small molecules, or pharmaceuticals can be engineered into the cDNA. Additionally, IK17 itself can be modified to improve stability, increase plasma elimination rate to decrease background and increased tissue uptake rate. The coding
20   sequence can be subjected to mutagenesis and screened against model compounds other than MDA-LDL to obtain antibodies that have slightly different specificities. Modifications can be made to optimize expression levels in the system of choice.

Conversion of IK17 into scFv

25   After analyzing the cDNA sequence of IK17, PCR primers were designed to create a human scFv from the parental pComb3H vector that harbors the cDNA for VL and VH genes of IK17. To amplify the variable gene rearrangements, one VH (400 base pair) amplification and one Vk (350 bp) amplification was performed. The products of each reaction were separately
30   pooled and ethanol precipitated. To perform an overlap PCR, aliquots of the Vk product were mixed with equal amounts of the VH product. The primers were created with identical sequences in the downstream portion of the Vk products

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and the upstream portion of the VH products to enable the creation of in-frame genes encoding scFv by overlap PCR. A 750 bp product for the Vk-linker-VH product was confirmed and was agarose gel size fractionated. The Vk-linker-VH sequence was then subcloned into Sfi site of prokaryotic expression vector

5   pARA, which has an arabinose inducible promoter for high level expression and a polyhistidine tag for affinity purification using a nickel column. The sequence of scFv IK17 has been determined and consists of entire Vk region and entire VH region (125 amino acids each) connected by a seven amino acid linker having a molecular weight of approximately 30 kD. Immunology testing has

10   shown that scFv IK17 has very similar binding properties as Fab IK17. In addition, scFv IK17 displays 50 to 500 greater binding activity to Cu-OxLDL and MDA-LDL than that of its parent Fab as assayed by chemiluminescent ELISA. *Labeling technique for noninvasive imaging.*

       IK17 can be genetically or chemically engineered to contain ^{99m}Tc binding

15   sites for nuclear scintigraphy imaging. *In vivo* SPECT imaging can be carried out in a number of hosts atherosclerosis. Because nuclear scintigraphy may not have ideal resolution to detect small lesions, IK17 can be labeled with gadolinium or echogenic liposomes for magnetic resonance and transvascular or intravascular ultrasound imaging, respectively.

20   *Conjugation of human MAbs to echogenic liposomes for transvascular enhancement of atherosclerotic lesions.*

       Recently, antibody-conjugated echogenic liposomes have been developed for site-specific intravascular (30 MHz) and transvascular (15 MHz) image enhancement. Anti-fibrinogen and anti-intercellular adhesion molecule-1

25   (anti-ICAM-1) antibodies have been conjugated to acoustically reflective liposomes and images obtained in animal models of thrombi and atherosclerotic lesions. These acoustic liposomes consist of a 60:8:2:30 molar mixture of phosphatidylcholine: phosphatidyl-ethanolamine: phosphatidylglycerol:cholesterol and are prepared by a dehydration/rehydration

30   mixture. They are multilamellar with well separated lipid bilayers and internal vesicles which confers echogenicity. Their mean size is ~800nm as measured by quasielastic light scattering. These liposomes are stable in circulation, do not

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trap gas, pass through pulmonary capillaries and retain their properties at 37°C,
even after conjugation with antibodies. The antibodies are thiolated with N-
succinimidyl-3-(2-pyridyldithio) propionate, reduced, and conjugated with the
liposomes by creating a thioether linkage between the antibody and
5   phospholipid. The conjugated antibodies are stable and have a long shelf half-
life. Atherosclerotic lesions are known to have increased permeability, which
enhances penetration into the deeper areas of the plaque. Plaques with the
thinnest "caps" - the endothelial/smooth muscle cell barrier overlying the
atheroma - and the ones most vulnerable to rupture, are also the most
10   permeable. Imaging is carried out as described below.

Gadolinium(Gd³⁺)-labeled scFv antibodies

       An alternative imaging method that provides enhanced resolution (<0.5
mm), magnetic resonance imaging (MRI), is evaluated by Gd^{3+}-labeling IK17 as
a contrast agent. MRI has the advantages of rapid acquisition, increased
15   resolution, absence of radioactivity and the ability to image the vessel wall
without interference from signal in the vessel lumen. However, because free
Gd^{3+} as a contrast agent is toxic, it is used in clinical MRI imaging bound to
diethylenetriaminepentaacetic acid (DTPA). Precedent exists for conjugating
Gd^{3+} to MAbs by reacting cyclic-diaminetriaminepentaacetic acid anhydride (c-
20   DTPA) with the MAb. Initial attempts using this technique were suboptimal but
subsequent studies have shown that polylysine-DTPA-Gd^{3+} coupled antibodies
can be used for tumour imaging with up to 30 Gd^{3+} ions conjugated without
significantly affecting antigen affinity. Previous studies using Gd^{3+} labeled MAbs
have either directly bound Gd^{3+} to available NH2 groups or chemically
25   conjugated polylysine. The natural site for coupling DTPA is limited in scFv
(single chain antibody) molecules. Therefore, we genetical fused several
clusters of polylysine groups (6-30 in length) to the N-terminal or C-terminal of
scFv MAb and react this with c-DTPA. Although other amino groups may
potentially react, the availability of polylysine in the tail of the molecule should
30   allow preferential site-directed labeling. The bioengineering of the polylysine site
was done by PCR using primers encoding six lysine residues and restriction site
for cloning at both 5' and 3' ends.

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Imaging with ^{99m}Tc-labeled MAb.

^{99m}Tc-labeling of oxidation specific antibodies has been previously described (Tsimikas et al., 1999). ^{99m}Tc-IK17 intravenously injected into atherosclerotic and normal mice and rabbits and is analyzed for the

5   pharmacokinetics, organ distribution and aortic plaque uptake. For *in vivo* imaging, 1-5 mCi are intravenously injected in hypercholesterolemia prone rabbits and imaging performed with a dual detector ADAC vertex model gamma camera set to a 20% window for ^{99m}Tc (VXUR collimator) equipped with ADAC Pegasys computer software. *In vivo* images planar (anterior, posterior and 45°

10   oblique positions) and SPECT will be acquired on a 256 X 256 X 12 matrix for a minimum of 1 X 10^6 counts at 10 minutes post injection. Repeat imaging is be performed for 3-500,000 counts at various timepoints based on the optimal target to background ratio derived from *in vivo* uptake data. Imaging studies using whole Mab often had a low signal to noise ratio due to the prolonged half-

15   life of the ^{99m}Tc-MAb in the circulation. Injections of the antigen, prior to imaging speed plasma clearance of the antibody, reducing the background for imaging. The use of Fab, scFv, or smaller fragments, can abrogate this problem under certain imaging conditions as the Fabs and scFvs have a very short half lifes (<30 minutes) and injection of antigen may not be required. When the signal to

20   noise ratio is not favorable, injections of MDA-LDL, Cu-OxLDL, or other appropriate antigen is injected to clear the background signal.

Imaging with Gd^{3+}-labeled MAb.

Labeling of Gd^{3+} to the antibody-DTPA complex has been previously described (Lister-James, et al, 1996; Wu et al, 1995). *In vivo* uptake assays are

25   carried out with ^{153}Gd-IK17 in mice and rabbits and the pharmacokinetics, biodistribution and aortic plaque uptake of IK17 is determined. *In vivo* imaging will then be performed in rabbits with a 1.5 T GE MRI scanner with a small surface coil

Transvascular enhancement of atherosclerotic lesions with echogenic liposomes

30   *conjugated with MAb.*

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Native IK17 or IK17 modified by the addition of cysteines to the C- or N-terminus of the protein is thiolated and conjugated to liposomes. A 12MHz imaging catheter (Acuson) is used for imaging (resolution <1mm). Rabbits prescreened for evidence of lesion and are injected with 2-4 ml of MAb-conjugated liposomes, unconjugated liposomes and normal saline. Videodensitometric analysis of liposome brightness is then carried out to assess uptake.

In vivo plaque uptake assay to determine presence or progression/regression of atherosclerotic lesions

To assess atherosclerotic lesions *in vivo*, labeled IK17 is injected into humans or animals having, or suspected of having, atherosclerotic disease. After a predetermined amount of time, dependent upon the stability of the labeling reagent, the type of imaging to be performed (local or whole body) and pharmacokinetic considerations, imaging is performed. To assess the efficacy of a treatment regimen, localized quantitative imaging is performed (e.g. with SPECT). To determine if disease is present anywhere in the body, full body imaging is performed. By the use of radioactive tracers on IK17, the progression or regression of plaques can be monitored quantitatively over the course of treatment with repeated imaging at desired intervals.

Intravascular imaging of lesions

Presently available methods of angiography could be combined with the use of labeled IK17 for enhanced imaging. The limitations of angiography were discussed above. Labeling of plaques before imaging would allow for the detection of smaller and more diffuse plaques that do not yet occlude the artery. IK17 labeled with ^{99m}Tc, gamma radiation or echogenic liposomes could be detected intravascularly by the use of catheters. This would increase the prognostic value of the method by providing a means to determine the composition of the lesion.

In vitro assay for the presence of atherosclerosis

To determine if OxLDL forms recognized by IK17 are present in the serum of hosts suspected of having atherosclerotic disease, a sensitive, double-layered

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sandwich chemiluminescent immunoassays were developed. For example, an antiserum which binds to apo B, a component of LDL, was coated onto the bottom of a microtiter plate. A series of dilutions of plasma is added to allow binding of LDL, the middle of the sandwich. After extensive washing, an appropriate dilution of IK17 Fab is added as the top layer of the sandwich. The presence of IK17 Fab is detected using an alkaline phosphatase linked Fab specific anti-human antibody which is in turn detected by a colormetric, luminescent or fluorescent assay. In a similar manner, an antiserum to human apoA1 can be used as the bottom layer to caputer HDL from plasma and allow the subsequent determination of IK17 epitopes in HDL. Finally, IK17 can be used as both top and bottom of the sandwich to obtain a measure of total IK17 epitopes.

Targeting of atherosclerotic drugs

One of the most challenging aspects of the development of pharmaceuticals is drug delivery. By the use of IK17, drugs for the treatment of atherosclerosis can be targeted to the required site of action, the atherosclerotic lesion. A panel of drugs are presently available for the treatment of atherosclerosis that work at the site of the lesion include thrombolytic agents, antioxidants, antimetalloproteinases, and immunomodulators. By targeting these drugs to their specific site of action, the active dose, and therefore the side effects, can be reduced. Additionally, active drugs with unfavorable pharmacokinetics can be linked to IK17 to improve their targeting to the plaque.

Development of atherosclerotic drugs

Immunization of LDLR$^{-/-}$ mice with OxLDL reduced the severity of the disease suggesting that antibodies of anti-OxLDL antibodies could be useful as therapeutic reagents. The Fab can be delivered directly or by means of a gene therapy vector as IK17 is expressed from a single gene.

A possible mechanism of action for the antibody is that by blocking the uptake of the OxLDL by macrophages, the formation of foam cells is inhibited and the progression of the disease is decreased. The binding site of IK17 for the

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OxLDL can be determined by direct structure determination or modeling, and used as a starting point for the development of small molecules to inhibit the uptake of OxLDL by macrophages.

Photodynamic therapy

5   IK17 can be labeled with photodynamic compounds that emit energy upon stimulation with an appropriate wavelength of light that can be administered by the use of a catheterized light source. Activation of the compound may ablate the atherosclerotic plaque or inhibit the growth of the plaque.

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Although an exemplary embodiment of the invention has been described above by way of example only, it will be understood by those skilled in the field that modifications may be made to the disclosed embodiment without departing from the scope of the invention, which is defined by the appended

10   claims.

        WE CLAIM:

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CLAIMS

1. A method of imaging atherosclerotic plaques in a host comprising:

introducing a diagnostically effective amount of detectably labeled human or humanized Mab or fragment thereof, Fab, scFv, or small molecule analog into the host vasculature, said antibody being specific for oxidation specific epitopes present in the core of atherosclerotic plaques, and binding to such epitopes *in vivo* at a detectably higher rate than the rate of binding to normal vasculature; and

determining whether the antibody binds to the vasculature, wherein the binding of said antibody to the vasculature is indicative of the presence of atherosclerotic plaques and the binding of said antibody to the vascular tissue is indicative of pathogenic, unstable plaques.

2. A method as in Claim 1 wherein the detectably labeled Fab or scFv is IK17.

3. A method as in Claim 1 wherein the size of the atherosclerotic plaque detected in the cardiovascular tissue is estimated as a correlate of the percent of the injected dose of detectably labeled antibody to another site in the body that does not contain atherosclerotic plaques.

4. A method as in Claim 1 wherein the imaging method is used as a means to monitor the progression or regression of atherosclerotic disease and is used as a prognostic indicator of the relative pathology of an atherosclerotic plaque.

5. A method of assaying for the presence of indicators of atherosclerosis in a host comprising assaying the serum of the patient for binding to a human or humanized Mab or fragment thereof, Fab, or scFv wherein the antibody is specific for oxidation specific epitopes present in atherosclerotic plaques wherein;

30   determining whether the serum contains factors that compete for the binding
      of the antibody to its antigen using an ELISA type assay wherein inhibition of

32   the binding of the antibody to its antigen indicates the presence of
      atherosclerotic indicators in the serum, or

34   the total population of HDL or LDL in serum are probed with the antibody in
      an ELISA type assay wherein binding of the antibody indicates the presence

36   of atherosclerotic indicators in the serum.

38   6. A method as in Claim 5 wherein the Fab or scFv is IK17.

40   7. A method of improving the delivery of therapeutic agents to the site of
      atherosclerotic lesions comprising linking theraputic agents to human or

42   humanized Mab or fragment thereof, Fab, ScFv, or small molecules wherein
      the antibody is specific for oxidation specific epitopes present in the core of

44   atherosclerotic plaques.

46   8. A method as in Claim 7 wherein the Fab or scFv is IK17.

48   9. A method as in Claim 7 wherein the therapeutic agent is delivered for the
      purpose of photodynamic therapy.

50

      10. A method of creating novel therapeutic agents which comprises agents

52   that block the uptake of OxLDL by macrophages by masking epitopes
      recognized by IK17.

54

      11. A method as in Claim 10 wherein the therapeutic agent is the antibody,

56   Fab or scFv itself wherein:
              the antibody is expressed recombinantly and administered to the host,

58   or
              the antibody is expressed by the host from a gene therapy vector

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60　　comprising the coding region of the Fab or scFv

62　　12. A method as in Claim 10 wherein the therapeutic agent is a small

　　　molecule that mimics the interaction of IK17 with oxidized epitopes to prevent

64　　their uptake by macrophages.

66　　13. A method for the modification of IK17 comprising the modification of the

　　　coding sequences for the light and heavy chains of the antibody or the

68　　addition of flanking sequences.

70　　14. A method in as in Claim 13 comprising addition of flaking sequences for

　　　creating a site or linker for modification with imaging or therapeutic reagents

72　　on IK17 or modifying the pharmacodynamic or pharmacokinetic properties of

　　　IK17 comprising altered stability, increased plasma elimination for reduction of

74　　background staining, and increased tissue uptake.

76　　15. A method as in Claim 13 comprising the modification if IK17 to alter the

　　　binding specificity of the Fab or scFv.

78

80　　16. An antibody containing the light and heavy chains comprised of the

　　　nucleotide sequences in the Appendix A attached hereto.

Organization Applicant

        Street : 300 Lakeside Dr, 22nd floor
        City : Oakland
        State : California
        Country : USA
        PostalCode : 94612-3550
        PhoneNumber : 510-748-6600
        FaxNumber : 510-748-6639
        EmailAddress :
<110> OrganizationName : Regents of the University of California

Application Project

<120> Title : Reagents and methods for diagnosing, imaging and treating
atherosclerotic disease
<130> AppFileReference : 6627-PA0045
<140> CurrentAppNumber : unknown
<141> CurrentFilingDate : 2000-10-26

Earlier Applications

<150> PriorAppNumber : 60/161,493
<151> PriorFilingDate : 1999-10-26

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1

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      SequenceDescription : Sequence of the heavy chain of the human
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Custom Codon

Sequence Name : IK17 heavy chain

Organization Applicant

      Street : 300 Lakeside Dr, 22nd floor
      City : Oakland
      State : California
      Country : USA
      PostalCode : 94612-3550
      PhoneNumber : 510-748-6600
      FaxNumber : 510-748-6639
      EmailAddress :
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Application Project

<120> Title : Reagents and Methods for diagnosing, imaging and treating atherosclerotic disease
<130> AppFileReference : 6627-PA0045
<140> CurrentAppNumber : unknown
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Earlier Applications

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<151> PriorFilingDate : 1999-10-26

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Custom Codon

Sequence Name : IK17 light chain

3

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(71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 9500 Gilman Drive, Mail Code 0910, La Jolla, CA 92037-0910 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): WITZTUM, Joseph [US/US]; 6912 Ofria Court, San Diego, CA 92093 (US). TSIMIKAS, Sotirios [US/US]; 2802 Amoldson Avenue, San Diego, CA 92122 (US). PALINSKI, Wulf [DE/US]; 4031-87 Porte Depalmes, San Diego, CA 92122-5150

(US). SHAW, Peter, X. [CA/US]; 10860 Caminito Arcada, San Diego, CA 92122-5150 (US).

(74) Agents: MCCLAIN, James, W. et al.; Brown, Martin, Haller & McClain LLP, 1660 Union Street, San Diego, CA 92101-2926 (US).

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(54) Title: REAGENTS AND METHODS FOR DIAGNOSING, IMAGING AND TREATING ATHEROSCLEROTIC DISEASE

(57) Abstract: The invention provides a novel human Mab Fab, cloned by phage display, and its use in diagnostic and therapeutic methods. In particular the invention provides a method for analyzing the OxLDL components of atherosclerotic plaques in vivo and a means to determine their relative pathology. As the method is based on a human Fab rather than a mouse Mab, the progress or regression of the disease may be monitored over time. The antibody may also be used for the analysis of surgical or serum samples ex vivo for the presence of OxLDL. The antibody may also be used to target therapeutic agents to the site of atherosclerotic plaques or may have use as a therapeutic agent itself.

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(7)   : G01N 33/53, 33/567

US CL   : 435/7.1; 435/7.21; 424/9.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, EMBASE, LIFESCI, WPIDS, BIOSIS, MEDLINE

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/21581 A1 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) 22 MAY 1998, see entire document	1, 3-5, 7, 9

☐   Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PATRICK J. NOLAN Telephone No. (703) 308-0196

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